UNITED NATIONS
INTERNATIONAL DRUG CONTROL PROGRAMME
Vienna

Recommended Methods for the Detection and Assay of Barbiturates and Benzodiazepines in Biological Specimens

MANUAL FOR USE
BY
NATIONAL LABORATORIES

UNITED NATIONS
New York, 1997

This publication has been based on the deliberations of the Expert Group Meeting on the Detection and Assay of Barbiturates and Benzodiazepines in Biological Specimens, held at Hong Kong from 13 to 17 November 1995. The views do not necessarily reflect those of the United Nations. This publication has not been formally edited.

ST/NAR/28

Contents

Chapter	*Page*
Introduction	1
A. Background	1
B. Purpose of the Manual	2
C. Use of the Manual	3
I. General Aspects of Assay of Controlled Drugs in Biological Specimens	4
A. Purpose and Strategy	4
B. General Guidelines for the Collection and Submission of Specimens for Drug Detection	4
1. Collection	5
2. Transport and Storage	7
3. Submission to the Laboratory	7
4. Request Form for Drug Analysis	7
C. Confidentiality of Results	8
D. Safety of Laboratory Personnel	8
E. Summary of Security Procedures	8
F. Methodology	9
1. Immunoassay Methods	9
2. Thin-Layer Chromatography (TLC)	10
3. Gas Chromatography (GC) and High Performance Liquid Chromatography (HPLC)	10
4. Gas Chromatography - Mass Spectrometry (GC-MS)	10
5. Sample Preparation	11
6. Quantitative Analysis	11
G. Quality Assurance	12
1. Internal Quality Control	12
2. External Quality Assessment	12
H. Interpretation	13
II. Recommended Methods for the Detection and Assay of Barbiturates in Biological Specimens	14
A. Introduction	14
B. Physical and Chemical Characteristics	16
C. Pharmacology	17
1. Current Uses of Barbiturates	17
2. Effects of Barbiturates	17
3. Development of Tolerance and Dependence	18
4. Potential for Abuse	18
D. Disposition	18
1. Routes of Metabolism	18
2. Urinary Excretion and Half-Life	19
E. Toxicology	21
1. Blood Concentration	21
2. Detection Time Limits in Urine	22
3. Interpretation of Results	22
(a) Urine	22

Chapter *Page*

(b) Blood, Serum and Plasma 22
(c) Interferences 23
F. Methods of Analysis 23
1. Introduction 23
2. Choice of Internal Standard 24
3. Extraction Procedures 25
(a) Liquid-Liquid Extraction 25
(i) Urine 25
(ii) Plasma and Blood 26
(b) Solid-Phase Extraction 26
(i) Urine 26
(ii) Blood 28
4. Screening Methods 30
(a) Immunoassay Methods 30
(b) Thin-Layer Chromatography 31
5. Confirmatory Methods 34
(a) Spectrophotometry 34
(b) Gas Chromatography 35
(i) Packed Column Technique 35
(ii) Capillary Column Technique 37
(iii) Detectors 40
(c) Gas Chromatography - Mass Spectrometry 40
(d) High Performance Liquid Chromatography 41

III. Recommended Methods for the Detection and Assay of Benzodiazepines in Biological Specimens 45
A. Introduction 45
B. Physical and Chemical Characteristics 52
C. Pharmacology 53
1. Current Uses of Benzodiazepines 53
2. Effects of the Benzodiazepines 53
3. Development of Tolerance and Dependence, Potential for Abuse 53
D. Disposition 54
1. Routes of Metabolism 54
2. Urinary Excretion and Half-Life 60
3. Target Analytes 63
E. Toxicology 65
1. Blood Concentration 66
2. Detection Time Limits in Urine 68
3. Interpretation of Results 68
(a) Urine 68
(b) Blood, Serum and Plasma 68
(c) Interferences 68
F. Methods of Analysis 69
1. Introduction 69
2. Choice of Internal Standard 70
3. Enzymatic Hydrolysis 71

Chapter *Page*

4. Extraction Procedures 71
   (a) Liquid-Liquid Extraction 72
   (b) Solid-Phase Extraction 73
5. Screening Methods 75
   (a) Immunoassay Methods 75
   (b) Thin-Layer Chromatography 77
6. Confirmatory Methods 81
   (a) Gas Chromatography 81
      (i) Packed Column Technique 81
      (ii) Capillary Gas Technique 84
      (iii) Detectors 85
   (b) Gas Chromatography - Mass Spectrometry 86
   (c) High Performance Liquid Chromatography 88
      (i) Blood 89
      (ii) Urine 90

References 93

Introduction

A. Background

Over the last decade there has been an enormous increase not only in the production and supply of illicit drugs, reflected by the huge and mounting quantities of drugs seized by national and international authorities, but also in the rate of drug abuse, i.e. illicit demand for drugs. Drugs seized are not only traditional drugs already under national and international control, but also include new illicit drugs or combinations of drugs prepared by chemists working in clandestine laboratories. At the same time there are reports of expanding misuse/abuse of drugs used for medical purposes, such as barbiturates and the benzodiazepines.

What used to be traditionally a problem of developed countries, is no longer confined to these countries. Drug abuse is now a global problem affecting developed and developing countries alike, and today no nation is free from this threat.

The extent and diversity of abuse have put increasing pressure on nations to intensify regulatory effort, in some cases with the introduction of stringent legislation which may have serious consequences on the individual charged with drug offences. Ultimately, the final outcome of such legislative procedures rests upon the results of laboratory tests. This has placed greater pressures on national laboratories which are now required not only to identify seized materials, but also to detect drug abuse. In addition, while in the past the laboratory was often only required to perform qualitative analysis, it is now required to also produce reliable quantitative results.

In the field of drug abuse, laboratories have now to be able to deal with more substances and to use methods of detection and analysis which are faster and yet, at the same time, are more accurate and specific. The analysis of biological specimens like urine and blood presents challenges because of the need to separate target substances from interferences in blood and urine which are complex biological matrices.

In addition, the international nature of the drug abuse problem requires speedy exchange of analytical data between laboratories as well as between the laboratories and law enforcement agencies on national and international levels. Development of internationally acceptable methods of detection and assay would contribute greatly towards achievement of these objectives.

An Expert Group in Kuala Lumpur in 1986 [1], while working on recommended methods for testing seized cannabis and amfetamine/metamfetamine, recognized that an issue of increasing importance to all member states was the development of methods for the analysis of abused drugs and their metabolites in body fluids. It was recommended that the United Nations should explore the most appropriate means of addressing this problem.

This proposal was endorsed by the Commission on Narcotic Drugs (CND) at its 32nd session in February 1987, which encouraged the United Nations Laboratory to extend its assistance to Member States by establishing and providing guidelines on methods of analysis of controlled substances in body fluids.

The International Conference on Drug Abuse and Illicit Trafficking (ICDAIT) had similarly suggested that "The Division of Narcotic Drugs, in collaboration with the World Health Organization (WHO) and the International Labour Organization (ILO), should promote and harmonize national efforts by developing internationally acceptable guidelines, criteria and methodologies for national testing programmes". The Conference also proposed "that a central source of reference standards of major drug metabolites should be established to serve national

laboratories [2].

In response to the suggestions of the CND and the ICDAIT, the former Division of Narcotic Drugs convened an Expert Group Meeting in 1987 on Guidelines for the Establishment of National Testing Programmes and Laboratories for Drugs of Abuse in Body Fluids. That Group recommended i) the "publication of follow-up working manuals on the subject to serve as guidelines for laboratory and programme development" and ii) "the setting up of an expert review group that would periodically review methodology and drug-testing procedures" [3].

The CND, at its tenth special session endorsed the recommendations of the Group and placed particular emphasis on "the development of recommended laboratory testing methods and international standard criteria for national body fluid testing programmes, including proficiency testing and method/procedure validation" [4].

In response to the Commision's request and at the invitation of the Government of Singapore, the former Division of Narcotic Drugs convened in 1989 an Expert Group Meeting on the Detection and Assay of Controlled Drugs in Biological Specimens and Recommended Methods for the Detection and Assay of Heroin/Morphine and Cannabinoids in Biological Specimens. A subsequent meeting was held in Madrid in 1990 on Methods for the Detection and Assay of Cocaine, Amphetamine, Methamphetamine, and Ring-Substituted Amphetamine Derivatives in Biological Specimens. At the invitation of the Government of Hong Kong, the UNDCP convened in 1995 an Expert Group Meeting on the Detection and Assay of Barbiturates and Benzodiazepines in Biological Specimens.

B. Purpose of the Manual

This manual, prepared by the Laboratory Section, Technical Services Division of the United Nations International Drug Control Programme, is part of a series related to the assay of drugs in biological specimens. It reflects the conclusions of the Expert Group Meeting held in Hong Kong in 1995 and has been designed to provide practical guidance to national authorities and analysts by describing recommended methods for use in forensic and toxicological laboratories for the detection and assay of barbiturates and benzodiazepines in biological specimens. Special emphasis has been laid on properly conducted and supervised sample collection, transport and storage, and strict maintenance of the chain-of-custody process. In performing assays on biological specimens, it is important that guidelines for the submission of samples are strictly adhered to. This is necessary because the results may have serious legal implications on the individual. In this context, the reader is referred to the United Nations manual on Recommended Guidelines for Quality Assurance and Good Laboratory Practices (ST/NAR/25) [5].

In selecting methods, the Expert Group was aware that many laboratories in existence today utilize methods which meet or may exceed legislative requirements. However, it was noted that there was great diversity with regard to the structure of national programmes and laboratory equipment and methodologies in the detection of drug abuse. In general, this manual is an attempt to help promote and harmonize national efforts by providing internationally acceptable guidelines and a selection of methods that may be used in the laboratories. More importantly, it is meant to provide assistance to laboratories which may not have access to sophisticated equipment and methods. Each method has been recommended as suitable and reliable. In the process of identifying these methods the Expert Group was aware that there are many other useful and acceptable methods.

C. Use of the Manual

Methods recommended in the manual are chosen on the basis of proven reliability, an important requisite if the results are to be used for legal or punitive objectives. The final choice of methodology and approach to analysis remains in the hands of the analyst working in his/her own country. This may necessarily depend on the availability of instrumentation, reference materials and trained personnel. However, it is recommended that for the purpose of establishing illicit consumption of drugs, two methods be used: an initial screening method (usually an immunoassay technique) followed by a confirmatory method using different chemical or physical principles (usually a chromatographic technique). Where only thin-layer chromatography (TLC) is available, it is suggested that a second thin-layer chromatography procedure utilizing a different solvent system be performed as well.

It is emphasized that whatever the methods selected, attention must be paid to proper equipment maintenance and environmental control, particularly for the transport and storage of specimens and unstable reagents, and that reliance is placed only on adequately trained and skilled personnel. Attention is also drawn to the importance of the availability of textbooks on drugs of abuse and analytical techniques. Furthermore, the analyst is expected to keep abreast of developments in the field of toxicological analysis by following current literature on the subject. Useful adjuncts to this manual would be the United Nations manuals on Recommended Methods for Testing Barbiturate Derivates under International Control (ST/NAR/18) [6] and Benzodiazepine Derivatives under International Control (ST/NAR/16) [7]. The reader is also referred to the United Nations manual on Recommended Methods for Detection and Assay of Heroin, Cannabinoids, Cocaine, Amphetamine, Methamphetamine and Ring-Substituted Amphetamine Derivatives in Biological Specimens (ST/NAR/27) [8].

The United Nations International Drug Control Programme (UNDCP) would welcome observations on the contents and usefulness of the present manual. Comments may be addressed to:

United Nations International Drug Control Programme
Technical Services Branch
Laboratory Section
United Nations Office at Vienna
Vienna International Centre
P.O. Box 500
A-1400 Vienna
Austria

I. General Aspects of Assay of Controlled Drugs in Biological Specimens

A. Purpose and Strategy

There are generally two purposes for analysis of biological fluids/specimens:

- For forensic purposes, i.e. the analysis of biological specimens for the presence of controlled drugs. A positive analytical result for a sample taken in this context would usually result in criminal proceedings and a punitive outcome for the defendant whose sample was analysed.

- For diagnostic, treatment and rehabilitative purposes, i.e. the analysis of samples from a clinical context to assess the cause of an intoxication or to determine if the sample donor has abstained from drug use within the previous few days. A positive analytical result in this context would not necessarily involve subsequent legal proceedings but might serve as a reliable indicator on which to base future medical treatment of the specimens donor.

Because punitive action may be the consequence of positive analytical results, procedures and methods used must follow strict standards based on principles of forensic toxicology. The generally recommended strategy is that an initial screening test should be performed to establish potential positive samples and this must be followed by a confirmatory test on such presumptive positive samples.

For the initial screening of specimens, laboratories should consider using immunoassay techniques such as radio immunoassay (RIA), enzyme immunoassay (EIA), fluorescence polarization immunoassay (FPIA) and latex agglutination inhibition (LAI). This should provide a rapid means of eliminating negative specimens. A positive result with immunoassay should then be followed by confirmation analysis using a method based on a different chemical or physical principle like thin-layer chromatography (TLC), high performance liquid chromatography (HPLC), gas chromatography (GC) or mass spectrometry (MS).

B. Guidelines for the Collection and Submission of Specimens for Drug Detection

The purpose of these guidelines is to describe procedures that will fulfil the necessary criteria in order to guarantee optimum validity of results. As recommended by the Brussels Expert Group [3], urine is the sample of choice for drugs-of-abuse testing. Apart from being readily accessible by non-invasive procedures, practically all drug metabolites are excreted in urine and the metabolites can be detected for a longer period than in blood. In this booklet blood methods have also been included since the Expert Group considered these a useful addition to urine testing for barbiturates and benzodiazepines. The use of other biological materials like hair and saliva for the present purposes, i.e. establishing the illicit consumption of drugs, has not yet been generally accepted.

In order to maintain the validity of analytical results in the forensic context, particular

care should be given to the **supervision** of specimen collection, transportation and storage.

Supervision must be done by trained personnel having a clear understanding of the legal implications of the procedure and this ought to be carried out by direct visual observation. Proper surveillance must be maintained at all times but every effort must be made to maintain the privacy and dignity of the individual. The supervisor should also ensure that no attempt is made to add contaminating or reactive substances to the urine.

When it is necessary to transport samples to an analytical laboratory, security and a clearly established chain of custody must be maintained.

These guidelines are applicable to situations where the collection of urine is conducted at sites which are located away from the analytical laboratories. This situation may not apply to all countries or different geographical locations within the same country. The guidelines should therefore be adapted or modified to suit the local situation, e.g. in the storage of specimens; if freezer facilities are not available, the analyst should incorporate stability checks in his quality control programme.

1. Collection

(i) Staff at the **collection site** are responsible for the collection, labelling, packaging and transportation of samples, ensuring that the collection and storage procedures have the proper documentation and necessary security methods.

(ii) All staff at the collection site must be provided with sufficient training to understand the collection process and its significance to the laboratory results.

(iii) The collection site must be supervised and witnessed by trained authorized personnel.

(iv) Suitable toilet facilities for the purpose of urine collection must be available before collection of urine is considered.

(v) The collection room must be surveyed for any substance which could be used to invalidate the sample and should be set up without soap dispensers or cleaning agents.

(vi) The urine specimen should be collected in duplicate in two 50 ml bottles. Each bottle should be filled at least 2/3 full. Plastic containers and rubber stoppers should be avoided whenever possible as non-polar drugs and their metabolites are very prone to absorb to some plastic and most rubber surfaces. If, for practical reasons, disposable plastic containers are used, individual laboratories should conduct tests to ensure that the plastic containers do not alter the composition or concentration of the drug(s) or metabolite(s) in the urine.

POSSIBLE WAYS TO INVALIDATE URINE SPECIMENS

1) Introducing various chemical substances to the specimen. Table salt, detergents or some commonly used household items such as hypochlorite (bleach) can destroy the drugs or affect the assay to generate false negative results.
2) In certain circumstances, adding illicit substances to the urine to produce positive results.
3) Placing a pinhole in the bottom of the container which results in leaks.
4) Using a fluid filled bulb placed under the arm, with a tube leading to the genital area. The bulb can be squeezed to release water or other substances which dilute or contaminate the urine.
5) Obtaining urine from friends not using drugs.
6) Scooping water from the WC into the collection container to dilute the urine.

(vii) Immediately after collection, the temperature (32°-38°C within 4 min) and the pH of the fresh urine specimen should be measured and recorded. If adulteration is suspected the laboratory should be notified.

(viii) The bottles should be securely stoppered, sealed and labelled. Steps should be taken to ensure that the integrity of the specimen is maintained, e.g. by use of a security seal consisting of sealing wax imprinted with a departmental seal or some other measure to indicate that tampering with the specimen has occurred. It is important that the donor witness the sealing of the bottles and sign or initial the seal or label.

(ix) Specimen labels should be affixed to the urine container and not to the lids. This will prevent accidental or intentional switching of specimens and/or identifying labels.

The label should contain at least the following information

Name: . . . . . .

I.D. number: . . . . . .

Date and time of collection: . . . . . .

Place of collection: . . . . . .

Name of person supervising the collection: . . . . . .

Drug(s) to be tested for: . . . . . .

Sample Number: . . . . . .

(x) Personnel details of each specimen donor are filled in a request form for analysis. The form will accompany the specimen to the laboratory.

(xi) The specimen donor should not be allowed to have any involvement in the post collection handling of the sample-labelling, packing and transportation to the laboratory.

(xii) Strict security should also be observed in the storage and dispensing of empty cups, request forms, labels and packing materials.

2. Transport and Storage

(i) After the request form has been completed, the specimen and request form are given to the dispatch person for transmission to the laboratory. Samples should be protected from direct light and heat during transportation and storage and should therefore be kept cold during transport, preferably in an insulated box containing ice or some other cooled packing.

It is important that specimens are kept cool and in the dark for the entire period between collection and analysis.

(ii) The designated dispatch person is responsible for transporting the specimens to the laboratory and maintaining appropriate chain of custody records for ensuring that the specimens are not tampered with during transit.

3. Submission to the Laboratory

(i) At the laboratory an authorized person should receive and carefully check the specimens and documents. One bottle of each urine specimen (or tube of blood) should be used for analysis and the other stored frozen for further analysis if necessary.

(ii) After ensuring the specimens and request form are in order, a written acknowledgement of receipt should be made, signed and given to the dispatch person.

(iii) The laboratory should maintain well-documented records and strict security to ensure integrity of specimens and confidentiality of results.

(iv) If the analysis is delayed beyond one or two days, specimens should be stored frozen, in a locked refrigerator. When frozen, specimens will generally be stable for several months.

4. Request Form for Drug Analysis

(i) A drug analysis request form which accompanies the specimens will allow the laboratory to check the individual specimens against the form to confirm the identity of the donor and that all specimens collected have actually reached the laboratory.

(ii) The form should contain, at least, identification data of the donor, of the person supervising the collection and of the dispatch person, specimen number, date and time

of collection, and the temperature and pH of the specimen at the time of collection.

(iii) Additional information which may be included on the form is a specification of the drugs for which the specimen is to be screened and a note of any suspicions concerning the validity of the specimen.

(iv) After completion, the form should be signed by an authorized person and stamped with an official seal.

C. Confidentiality of Results

It is important to maintain complete security and confidentiality at all times.

(i) Any information related to the donor and to the results of the analysis must be kept locked and secure.

(ii) Reports should be accessible only to authorized persons.

D. Safety of Laboratory Personnel

The handling of biological materials exposes personnel to infection hazards from, amongst others, hepatitis and AIDS. All personnel should therefore take the necessary precautions and adhere to safety procedures such as wearing gloves and other protective clothing.

E. Summary of Security Procedures

(i) In addition to the specimens, strict security should also be observed in the storage and dispensing of empty cups, request forms, labels and packing materials.

(ii) The specimen donor should not be allowed to have any involvement in the post collection handling of the specimen - labelling, packing and transportation to the laboratory.

(iii) It is important that the donor witnesses the sealing of the container and signs or initials the seal or label.

(iv) Accurate and complete records of all individuals involved in the urine collection, storage and transport should be maintained.

(v) Specimen labels should be affixed to the urine (or blood) container and not to the lid. This will prevent accidental or intentional switching of specimens and/or identifying labels.

(vi) Information on sample donors and results should be kept strictly confidential and be accessible only to authorized persons.

F. Methodology

As recommended earlier, both screening and confirmatory tests are required.

A screening test should be able to identify potential positives with a high degree of reliability and should be sensitive, rapid and inexpensive. These criteria are generally met by immunoassays. However, the antibodies used in immunoassays have relatively low specificities and may result in cross-reactivity. All positive results obtained by an immunoassay screen should be confirmed by a second test based on different chemical principles. Thin-layer chromatography (TLC) may also be used as a screening test.

Confirmatory tests should be at least as sensitive as, but more specific than, screening tests. They generally involve chromatographic techniques and may include TLC, gas chromatography (GC), high performance liquid chromatography (HPLC) and gas chromatography - mass spectrometry (GC-MS).

1. Immunoassay Methods

Immunoassays are the methods of choice when large numbers of specimens must be assayed within a limited time. Several immunoassay kits are available commercially for the screening of drugs of abuse. The most commonly used methods are radio immunoassay (RIA), enzyme immunoassay (EIA), fluorescence polarization immunoassay (FPIA) and latex agglutination inhibition (LAI). RIA, FPIA and EIA require instrumentation that is relatively expensive. Any of the above assays may be used by laboratories which have access to them.

The choice of technique would depend in most instances on the workload (number of specimens per day) handled by the laboratory. The enzyme immunoassay and radio immunoassay techniques for instance are available in either the single- or multi-test versions. For laboratories with small numbers of specimens, the single-test versions, or latex agglutination inhibition may be utilized but are expensive when considered in terms of the cost of analysis per specimen. For large workloads, the multi-test enzyme immunoassay or the fluorescence polarization immunoassay are more appropriate.

Adequate consideration must be given to equipment maintenance, environmental control (temperature stability), and supply and (cold) storage of relatively unstable reagents to minimize inaccuracies in results. False results may also be the consequence of specimen adulteration, for example by addition of pH-modifying (vinegar, ascorbic acid, lemon juice, lime solvent etc.), oxidizing (sodium hypochlorite), surface-active (detergent, soap etc.) and enzyme-desactivating (glutaraldehyde) agents, medicaments (such as tetrahydrozoline containing eye or nose drops), sweeteners (saccharin) and sodium chloride. Most popular manipulations are endogenic (excessive drinking, use of diuretics) and exogenic dilution (addition of water) as well as exchange or substitution of urine specimen.

Training and experience requirements may be less for some immunoassay techniques, which facilitates laboratory staffing, but supervising analysts with extensive experience of the techniques should be present.

Some of the characteristics of the main immunoassays are summarized in Table I.1.

Table I.1 General characteristics of immunoassays

Feature	EIA	FPIA	RIA	LAI
Requirement for special instrumentation	Yes	Yes	Yes	No
Stability of reagents	Months	Months	3-4 weeks	> 1 year
Costs of reagents	+++[a] ++[b]	++(+)	+	+++
Possibility of automation	Yes	Yes	Yes	No
Tests per technician per 8 h shift	100-400[b]	250-300	200-400	200-350

[a] Single test (st) [b] Drug abuse urine assay (dau)

2. Thin-Layer Chromatography (TLC)

TLC methods are inexpensive in terms of capital equipment and other initial set-up costs. They are labour-intensive, generally less sensitive than other techniques and require considerable experience for accurate application due to the subjective nature of their interpretation. They are recommended as a confirmation assay for immunoassay screening results and as the primary test where labour expenses are of less importance than capital outlays, but where adequately trained staff are available.

In situations where resources limit the laboratory to TLC methodology alone, the assay result should not be used as the sole proof of drug presence or use when the consequences impact severely on the individual. In the absence of more sophisticated equipment, an acceptable solution can be a confirmation using at least one alternative TLC solvent system and/or detection reagent.

3. Gas Chromatography (GC) and High Performance Liquid Chromatography (HPLC)

GC and HPLC offer high sensitivity and specificity for confirmation of presumptive positive results in screening assays. The equipment is, however, relatively expensive in comparison to TLC or immunoassay and training and experience for these highly technical systems are critical.

4. Gas Chromatography - Mass Spectrometry (GC-MS)

GC-MS is the most sensitive method available for confirmation of drug presence in a specimen. It requires the greatest outlay in capital, training and maintenance costs. It is the method least likely to be challenged in court and should be considered as a necessary and important asset in national programmes where the control laboratory will be the final source of confirmation for questioned assays.

5. Sample Preparation

In general, very little sample preparation is required for initial immunoassay tests. It is unnecessary to hydrolyse the urine specimens because immunoassays measure both the free and conjugated forms of the drug and/or metabolites. It may be necessary to adjust the pH or centrifuge the urine to remove turbidity. For optimum results, the manufacturer's instructions should be followed.

For chromatographic procedures, good sample preparation is extremely important. This is necessary because urine (and blood) is a complex containing a mixture of large amounts of numerous organic and inorganic compounds in which the specific target analyte is found in minute amounts. Sample preparation usually involves hydrolysis of urine and the extraction and purification of the analyte.

The procedure should be efficient, since a good recovery is necessary to extract the small amounts present, and selective, to ensure that interfering substances in the specimen are removed.

Sample preparation for GC and GC-MS often involves the preparation of chemical derivatives of the target analytes. Although this additional step may require additional time and expense because of the reagents used, nevertheless derivatization is frequently recommended for the following reasons:

- It can provide greater sensitivity.
- The derivatized compounds may be more thermally stable.
- Chromatographic properties may be improved, i.e. peak shape, retention times and separations.
- The mass spectra contain ions which are more suitable for GC-MS in the selected ion monitoring (SIM) than those of the underivatized forms.

6. Quantitative Analysis

For the purpose of establishing the illicit use of drugs, it is not absolutely necessary to use quantitative analytical methods. However, there are many advantages in measuring the quantities of drugs and their metabolites identified in the screening method(s), particularly with respect to problems of interpretation.

Chromatographic methods generally give reliable quantification of analytes. TLC methods may be used as a quantitative procedure but would require a plate scanner or densitometer and may not be reliable or cost effective. Also, immunoassay methods generally do not give reliable quantification in this context because of the inherent possibilities of unidentified cross-reacting substances being present in the specimen.

Quantitative analysis by GC, HPLC or GC-MS requires an internal standard to be added to the specimen prior to extraction. An internal standard also permits the measurement of relative retention time. Internal standards should resemble the target analytes such that they can be extracted, derivatized and analysed under the same conditions as the target analytes, but be readily distinguished from them during the chromatographic procedure. Care must be taken, however, to avoid using substances which might occur in the specimen such as other drugs or endogenous materials.

For quantitative analysis performed by GC-MS a deuterium-labelled analogue of the analyte is usually the best choice as internal standard. However, deuterium-labelled analogues are expensive and may not be readily available. Other analogues of the target compound are also generally satisfactory as internal standards.

If the decision is taken to establish quantitative methods, an immediate consequence is the need to verify the method with respect to accuracy and precision, as discussed below in Chapter I.G.. The coefficient of variation of a chromatographic method should be less than 10% and preferably less than 5%.

The concentration of an analyte can be calculated using the general formula:

$$\text{Concentration of analyte } X = \left[\frac{A_X/A_{IS} \text{ in sample chromatogramme}}{A_{RS}/A_{IS} \text{ in standard chromatogramme}} \right] \cdot C_{RS}$$

Where:

A_X = Peak area for analyte X obtained from the sample chromatogramme

A_{IS} in sample chromatogramme = Peak area of internal standard obtained from the sample chromatogramme

A_{RS} = Peak area for reference standard obtained from the standard chromatogramme

A_{IS} in standard chromatogramme = Peak area of internal standard obtained from the standard chromatogramme

C_{RS} = Concentration of analyte X in the reference standard solution

G. Quality Assurance

Properly trained and skilled personnel are basic requisites for reliability of results. Adherence to good laboratory procedures and practices (GLP), standard operation procedures (SOP) and regular retraining of staff will help maintain quality and reliability of the laboratory.

1. Internal Quality Control

A good and well-documented quality assurance programme must be an integral part of the drug laboratory set-up and it should at least incorporate some means of assessing the accuracy and precision of all analyses done. The precision of methods should be assessed either by multiple analyses of individual specimens and/or inclusion of a sufficient number of quality control specimens (with different concentrations of the drug or metabolite in the relevant body fluid). This will enable the analyst to conduct statistical evaluations of precision within batches over a period of time.

2. External Quality Assessment

Where possible, the laboratory should participate in an external proficiency programme. Ideally, such a programme should be conducted by an independent external agency such as the United Nations and laboratories in Member States should be invited to participate. In the absence of such a programme, laboratories within a country can adopt the following strategy:

(i) Inter-laboratory proficiency programme: this is done by laboratories submitting specimens to each other for analysis and checking on each other's performance.

(ii) The main laboratory should be designated as the reference laboratory. This centre should send specimens containing different concentrations of the analyte(s) to all laboratories for analysis. Results of analyses would then be evaluated by the reference laboratory.

H. Interpretation of Results

The qualitative or quantitative analysis of a biological specimen will provide evidence that a subject has or has not used a controlled drug. The presence of metabolites can show that a drug has been absorbed into the body.

A positive result on initial screening means that a drug or metabolite is present in the specimen at a concentration above or equal to the cut-off concentration. Elimination from the body and drug concentrations in urine and blood depend on such factors as the route of administration, frequency and duration of use, rate of drug metabolism, subject's physical condition, collection time and fluid intake etc.. It is important to note, however, that the concentration of drug in urine can in no way be related to the level of impairment.

II. Recommended Methods for the Detection and Assay of Barbiturates in Biological Specimens

A. Introduction

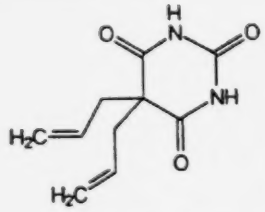
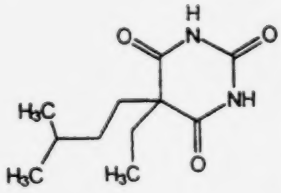
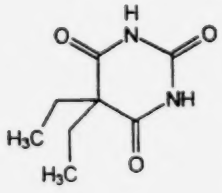
Barbiturates are a relatively homogeneous group of synthetic drugs. They are therapeutically used as sedatives, hypnotics, anesthetics and anticonvulsants in relatively high doses. For many of their uses they have been replaced by the benzodiazepines in many of the developed countries.

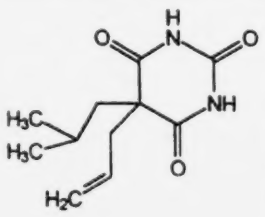
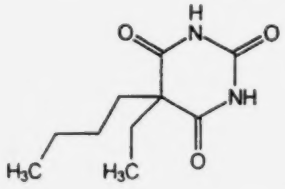
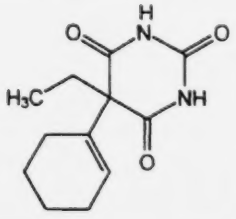
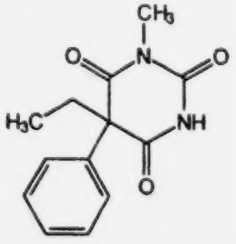
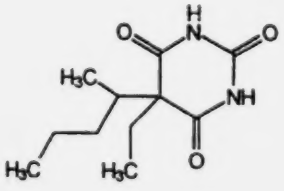
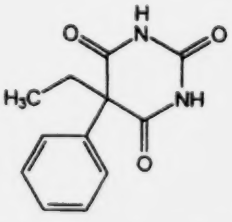
Reportably, over 2500 barbiturates have been synthesized. More than 50 are presently marketed for clinical use throughout the world. Barbiturates often occur as mixtures with other barbiturates (amobarbital/secobarbital) as well as with other substances (caffeine, acetyl salicylate, ephedrine, theophylline, codeine).

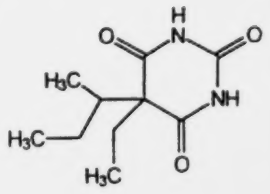
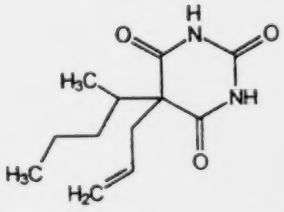
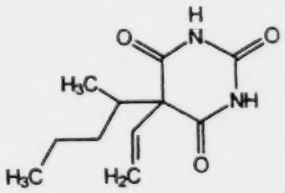
Abuse/misuse of barbiturates is internationally widespread which means that any forensic laboratory may encounter a range of these compounds. Virtually all of the barbiturates in the illicit market result from diversion from legitimate sources and there is no evidence of clandestine manufacture.

Out of the presently marketed barbiturates twelve are subject to international control under the 1971 Convention on Psychotropic Substances.

Table II.1 Barbiturates under international control

Allobarbital		$C_{10}H_{12}N_2O_3$ M.W. 208.2 pKa 7.8 Schedule IV
Amobarbital		$C_{11}H_{18}N_2O_3$ M.W. 226.3 pKa 7.9 log P (octanol/pH 7.4) 1.6 Schedule III
Barbital		$C_8H_{12}N_2O_3$ M.W. 184.2 pKa 8.0 Schedule IV

Butalbital		$C_{11}H_{16}N_2O_3$ M.W. 224.3 pKa 7.6 Schedule III
Butobarbital		$C_{10}H_{16}N_2O_3$ M.W. 212.2 pKa 8.0 log P (octanol/pH 7.4) 1.7 Schedule IV
Cyclobarbital		$C_{12}H_{16}N_2O_3$ M.W. 236.3 pKa 7.6 log P (octanol/pH 7.4) 1.8 Schedule III
Methylphenobarbital		$C_{13}H_{14}N_2O_3$ M.W. 246.3 pKa 7.8 Schedule IV
Pentobarbital		$C_{11}H_{18}N_2O_3$ M.W. 226.3 pKa 8.0 log P (octanol/pH 7.4) 1.9 Schedule III
Phenobarbital		$C_{12}H_{12}N_2O_3$ M.W. 232.2 pKa 7.4 log P (octanol/pH 7.4) 1.4 Schedule IV

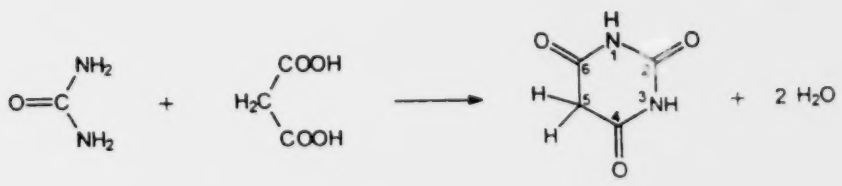
Secbutabarbital		$C_{10}H_{16}N_2O_3$ M.W. 212.2 pKa 8.0 log P (octanol/pH 7.4) 1.3 Schedule IV
Secobarbital		$C_{12}H_{18}N_2O_3$ M.W. 238.3 pKa 7.9 Schedule II
Vinylbital		$C_{11}H_{16}N_2O_3$ M.W. 224.3 Schedule IV

From INCB statistics [9], the significant barbiturates in the last decade have been amobarbital, butalbital, cyclobarbital, pentobarbital and phenobarbital.

Analysts should be aware of the particular barbiturates commonly available in their area. For information on their characteristics and methodologies for their identification and analysis reference should be made to the United Nations manual on Recommended Methods for Testing Barbiturate Derivates under International Control (ST/NAR/18) [6] as well as to national pharmacopoeias. The Multilingual Dictionary of Narcotic Drugs and Psychotropic Substances under International Control (ST/NAR/1/REV.1) [10] published by UNDCP includes a listing of many brand names and other synonyms for barbiturates under international control.

B. Physical and Chemical Characteristics

The parent compound of the barbiturate group of drugs is barbituric acid, which is formed by the condensation of urea and malonic acid:



The two hydrogen atoms at position 5 can be replaced by different organic radicals to form a large number of barbiturate compounds, i.e. the 5,5'-disubstituted barbiturates. In some cases also the hydrogen atom at position 1 is replaced by an alkyl group (e.g. methylphenobarbital). The oxygen atom linked to the carbon in position 2 can be replaced by sulfur, i.e. the thiobarbiturates (e.g. thiopental).

In the free acid form, barbiturates are soluble in most organic solvents such as ethyl ether, ethyl acetate, chloroform and methanol, but are insoluble in water. Amobarbital, pentobarbital, phenobarbital, secbutabarbital and secobarbital are available as sodium salts, cyclobarbital as a calcium salt. These salts are generally insoluble in ethyl ether, ethyl acetate and chloroform, but are soluble in methanol and water.

C. Pharmacology

The therapeutic use of barbiturates has declined in recent years because they do not have specificity with respect to their central nervous system (CNS) effects. They have a lower therapeutic safety margin than alternatives such as the benzodiazepines and tolerance occurs more often than with benzodiazepines. They have a greater abuse liability and exhibit a significant number of drug interactions [11,12].

1. Current Uses of Barbiturates

Current uses include:

- as hypnotics and sedatives - for example phenobarbital is used in a variety of drug mixtures for gastrointestinal disorders, urethral inflammation, hypertension, asthma and coronary artery disease;
- as antagonists for unwanted side-effects of central stimulants such as to ephedrine;
- phenobarbital is used as an anticonvulsant in the treatment of epilepsy;
- as intravenous anesthetics (most often thiopental (or also known as Pentothal), which is not controlled under the 1971 Convention).

2. Effects of Barbiturates

The barbiturates can cause all degrees of depression of the CNS from mild sedation to general anaesthesia. Some have selective anticonvulsant activity. They show a lower anxiolytic effect than benzodiazepines compared to the sedative effect. They can exert a euphoriant effect similar to that of morphine, but do not have any significant effect on the perception of pain. They cannot be relied on to produce sleep or sedation in the presence of even moderate pain. In some users, the presence of pain can cause a paradoxical excitement. When injected by drug abusers, they can cause violent behaviour [12].

3. Development of Tolerance and Dependence

Tolerance[1] to the effects of barbiturates occurs during the chronic administration of barbiturates, over a period of weeks to months. Drug metabolism is also induced[2], but within a few days to a week. It is important to note that tolerance to the effects of barbiturates does not significantly elevate the blood concentration that can cause toxicity.

Physical dependence that accompanies the development of tolerance and sudden withdrawal from the drugs in a dependent individual can be dangerous, resulting in delirium, convulsions and occasionally death. Phenobarbital readily crosses the blood/placental barrier and is associated with a higher than normal incidence of birth abnormalities in infants born to mothers using barbiturates. Withdrawal symptoms may be present in infants born to mothers dependant on barbiturates.

4. Potential for Abuse

As a result of their sedative and hypnotic properties, barbiturates are potentially habit-forming, with tolerance, and psychological and physiological dependence occurring with continued use. Patients who develop such a dependence will frequently increase the dosage or decrease the dosage interval without consulting a physician. Deliberate abuse of barbiturates is relatively uncommon, at least in comparison with other illicit drugs. Barbiturates (and other drugs) may be added to folk or patent medicines for their anxiolytic properties and may be consumed unwittingly [13]. The use of barbiturates, often in conjunction with the use of a plastic bag placed over the head promoting suicide, has also been advocated in literature.

D. Disposition

1. Routes of Metabolism

Most barbiturates are biotransformed in the body to inactive metabolites by one of the four possible routes shown in Figures II.1 and II.2 below.

These routes are:

(i) oxidation of the C^5 substituents with formation of alcohols, phenols, ketones and carboxylic acids which may appear in the urine both as free compounds and as glucuronic acid conjugates;

(ii) desalkylation of the *N*-alkyl groups;

(iii) desulfuration of the S^2 group of thiobarbiturates;

(iv) destruction of the barbituric acid ring between N^1 and C^6.

Of particular relevance in the forensic context is the partial metabolism of thiopental to pentobarbital (Figure II.2). Consequently, if pentobarbital is detected in urine, the use of thiopental should be excluded.

---

[1] Tolerance refers to a term used to describe an adaptation to the effects of a drug, usually following repeated use, requiring larger doses to exhibit a standard response.

[2] This refers to an accelerated metabolism produced by an effect of barbiturates on enzyme activity.

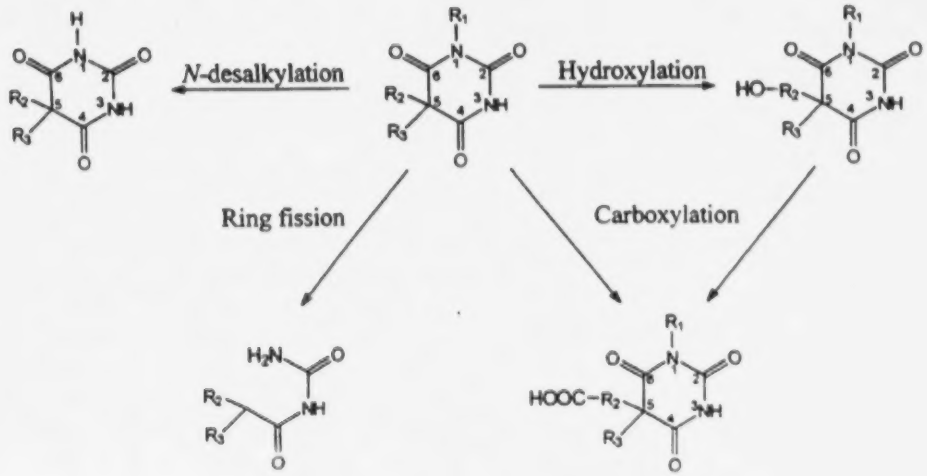
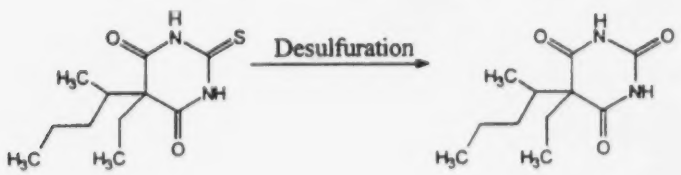


Figure II.1 General routes of metabolism of barbiturates



Thiopental Pentobarbital

Figure II.2 Metabolism of thiopental to pentobarbital.

2. Urinary Excretion and Half-Life

A summary of the main metabolites and some relevant pharmacokinetic data are given in Table II.2.

Table II.2 Metabolism, half-lives and excretion data for barbiturates under international control

Barbiturate	Parent compound excreted	Known metabolites	Plasma $t_{½}$ (h)
Allobarbital	10-35% slowly excreted over 1 week	Not known	40-48
Amobarbital	1-3%	- 3'-hydroxyamobarbital (30-50%) - *N*-glucopyranosylamobarbital (up to 30%) - 5-(3'-carboxybutyl)-5-ethylbarbituric acid (5%)	8-40 mean 24

Barbiturate	Parent compound excreted	Known metabolites	Plasma $t_{½}$ (h)
Barbital	almost all, 95% (2% in 8h, 16% in 32h)	(also produced as a metabolite of metharbital)	about 48
Butalbital	5%	- 5-(2,3-dihydroxypropyl) metabolite (20-60%) - 5-(3-hydroxy-2-methyl-1-propyl) metabolite (10%)	30-88
Butobarbital	5-9%	- 3'-hydroxybutobarbital (22-28%) - 3'-oxobutobarbital (14-18%) - 3'-carboxypropyl metabolite (4-8%)	about 40
Cyclobarbital	less than 10%	- 5-(3-oxocylcohex-1-enyl)-5-ethylbarbituric acid	8-17 mean 12
Methylphenobarbital	less than 2%	- phenobarbital (up to 10%) - *p*-hydroxymethylphenobarbital (30-35%, as free and conjugated forms) - *p*-hydroxyphenobarbital (small amounts) - 5-ethyl-5-(4-hydroxy-3-methoxyphenyl)-barbituric acid (small amounts) - 5-ethyl-5-(4-hydroxy-3-methoxyphenyl)-1-methylbarbituric acid (small amounts)	50
Pentobarbital [14-16]	less than 1%	- 3'-hydroxypentobarbital (7% *d*-, 30% *l*-) - 3'-oxypentobarbital (7-14%) - 3'-carboxy metabolite (10-15%) - *N*-glucoside conjugate (13%)	up to 48 mean 27
Phenobarbital [17-20]	25-67%	- *p*-hydroxyphenobarbital (17%, half of which is conjugated) - *N*-glucopyranosylphenobarbital (up to 30%) - two dihydrodiol metabolites - hydroxymethylphenobarbital	50-150 mean 100
Secbutabarbital	5-9%	- 5-(2-carboxy-1-methylethyl)-5-ethyl-barbituric acid (30%) - 2'-hydroxysecbutabarbital (3%) - 2'-oxosecbutabarbital (1%)	34-42
Secobarbital [16]	less than 5%	- 3'-hydroxysecobarbital (two diastereo-isomeric forms) - 5-allyl-5-(3-carboxy-1-methylpropyl)-barbituric acid (4%) - 5-(2,3-dihydroxypropyl)secobarbital (4%) - 3'-oxosecobarbital (3%)	19-34 mean 25
Vinylbital [21]	5%	- 3-hydroxy metabolite	18-33

Notes

1. Data derives from references cited as well as references 21 and 23.
2. The % parent drug excreted may be higher in overdose cases.
3. Only limited information is available on the metabolism and excretion of vinylbital.

E. Toxicology

1. Blood Concentration

Data on therapeutic and toxic blood (and plasma) concentrations can be found in Stead and Moffat [22], Baselt and Cravey [23], Clarke's [21] and TIAFT Bulletins [24]. Table II.3 summarizes these data.

Table II.3 Therapeutic and toxic blood concentrations of barbiturates

Barbiturate	Maximum therapeutic level (mg/l)	Minimum level for toxicity (mg/l)
Allobarbital	40	50
Amobarbital	12	10
Barbital	30	20
Butalbital	10	10
Butobarbital	15	14
Cyclobarbital	10	8
Methylphenobarbital	15	40
Pentobarbital	10	5
Phenobarbital	40	3
Secbutabarbital	14	20
Secobarbital	10	10
Vinylbital	10	5

Precautionary Note. The concentrations listed in Table II.3 are a guide only. Blood (or plasma) concentrations should be interpreted with caution. The toxic effects of barbiturates are dependent on the degree of tolerance developed by the person, the presence of any natural disease, particularly respiratory or cardiovascular, and the presence of any other psychoactive drugs, e.g. alcohol and other CNS depressant drugs (narcotics, benzodiazepines etc). Such factors usually reduce the concentration required to elicit a toxic response. An example frequently encountered is the high tolerance developed by epileptics to phenobarbital. The range of blood concentrations encountered in patients treated regularly with phenobarbital may overlap with the toxic range. Urine concentrations should not be interpreted with respect to dose and likely pharmacological effects.

Intravenous use of these drugs may lead to a lower blood concentration of drugs which causes a fatality than would generally be found following oral administration. This is especially true for phenobarbital, but this may also be true for other barbiturates.

2. Detection Time Limits in Urine

The time interval over which a barbiturate can be detected in urine is highly variable. Several factors contribute to the time interval of detection, including:

(i) dose of the drug,
(ii) chronic versus acute administration,
(iii) the co-ingestion of other drugs which may impair or enhance metabolism of the barbiturate,
(iv) the analytical methodology used,
(v) differences in the metabolism and excretion of drugs classified as barbiturates,
(vi) the pH of the urine.

In general, barbiturates can generally be detected in urine for approximately 24 hours after use for short-acting barbiturates such as pentobarbital and secobarbital, but much longer for the long-acting barbiturates such as phenobarbital (up to 14 days or more [25]).

3. Interpretation of Results

(a) Urine

Urine concentrations cannot usually be related to a particular drug dose or time since last dose, since urinary excretion depends on the volume of water excreted, creatinine clearance (kidney function), and time since last dose.

Individuals also vary greatly in their ability to metabolise drugs, hence there are variable amounts of parent drug excreted into urine. Most of the barbiturates are extensively metabolised (see Table II.2).

Some barbiturates such as barbital, pentobarbital and phenobarbital are metabolites of other barbiturates and other drugs such as primidone, although they can be used as drugs in their own right. This possibility should be considered in any interpretation of the presence of barbiturates detected in urine.

It is not possible to infer a likely degree of intoxication from urine concentrations.

(b) Blood, Serum and Plasma

Blood (or serum or plasma) can be used to obtain an estimate of the degree of drug use. Table II.3 illustrates the usual concentrations associated with therapeutic use and those possibly associated with the development of toxic symptoms.

There is, however, some overlap between these two ranges. Toxic symptoms are more likely to develop at the upper end of the therapeutic range in persons not normally exposed to barbiturates or in those persons using other drugs acting on the CNS (alcohol, benzodiazepines, narcotics etc.) and in those persons with significant respiratory and/or cardiovascular disease.

On the other hand, persons who have developed some degree of tolerance may not develop toxic symptoms unless rather higher concentrations are attained.

Toxic symptoms include slurred speech, unsteadiness and poor coordination. Serious toxicity usually manifests as convulsions and disturbance of breathing, leading to coma and death.

Barbiturates have a narrow therapeutic index (i.e. difference between therapeutic and toxic concentrations) and death can occur with excessive use of barbiturates at concentrations often not much higher than the upper limit of the therapeutic range.

(c) Interferences

1. Barbiturate-like ions may be observed in the GC-MS analysis of specimens from decomposed bodies, although barbiturates are not present.
2. Other drugs may be metabolised to phenobarbital, including methylphenobarbital and primidone, and pentobarbital is produced, in part, from thiopental.

F. Methods of Analysis

1. Introduction

The following chapters concern different aspects of methods for the analysis of barbiturates in biological specimens. The specimens covered are urine, the recommended specimen for detecting drug use and abuse [3], and blood, serum and plasma, which are used for therapeutic drug monitoring and detection of drug use in the clinical setting, the assessment of drug-induced impairment in, for example, the traffic safety context, and for assessment of the cause of death in drug-related fatalities in forensic medicine.

The procedures described in this manual are intended to guide the laboratory in the appropriate selection of suitable assay procedures. Many of the barbiturates available, but not under international control, may also be assayed by the procedures described.

To improve the readability of the text, plasma and serum are treated as synonymous although they are hematologically distinct.

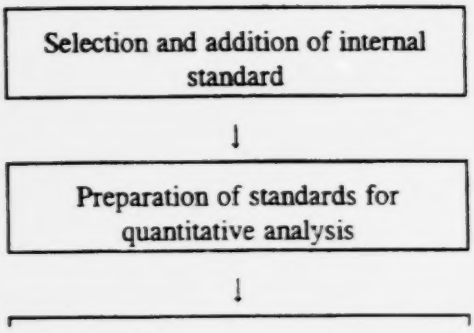
When a complete method for the analysis of one or more barbiturates is required for a particular biological specimen such as urine or blood, appropriate selections should be made, as required, from the information provided in subsequent chapters. Alternatives have been provided to assist the analyst, who may not have access to all of the materials and equipment necessary for any complete method published in the literature. Each of these individual components of a method are recommended as being suitable for their specific purpose and can be depended upon to work reliably.

However, as stated in Chapter I., the ultimate choice of methodology depends inter alia on the type of specimen to be analysed, the context and purpose of the analysis (clinical or forensic), the facilities available in the laboratory and the experience of the laboratory staff in this field of analytical chemistry. The analyst has the final decision on the choice of which is the most appropriate method, for only the analyst is in possession of all the relevant facts concerning the specimen which is to be analysed.

Above all, it is imperative that any method, constructed from components listed below, is evaluated by the analyst testing it with standards made up in the same sample matrix as the specimens to be analysed and establishing if the method is suitable for the intended purpose.

A method may contain some or all of the components listed below. The decision on whether or not they should be included in a method depends on the type of specimen (urine or blood), the type of analysis (qualitative or quantitative), the purpose of the analysis (screening or confirmatory) and the type of equipment which will be used in the analysis

(immunoassay, TLC, GC, GC-MS or HPLC). The latter (analytical equipment) determines the sensitivity and specificity of the analysis and, as a corollary, the amount of analyte which must be available at the end step. This consequently determines the volume of specimen required for the analysis.



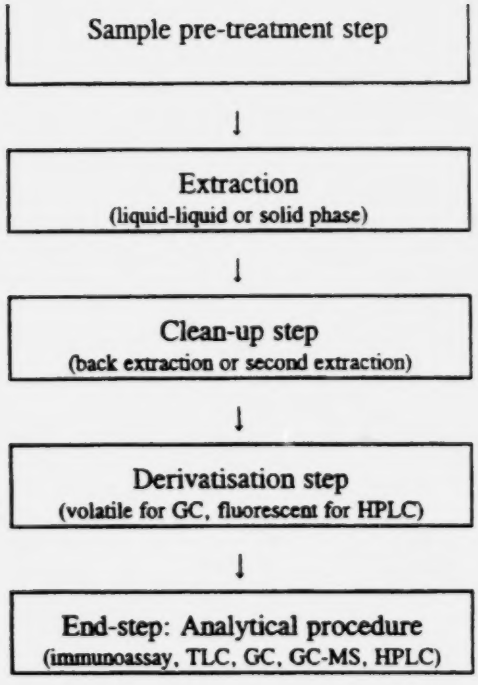


Figure II.3 Flow diagramme for the analysis of barbiturates in biological specimens

2. Choice of Internal Standard

The extraction procedure normally includes the addition of an internal standard. Suitable internal standards must take into account the method of extraction and the end-step procedure for detection, confirmation and quantitation. Suitable internal standards in all cases are barbiturate derivatives which are unlikely to be found in specimens because they are not used as drugs or are no longer prescribed in that country.

Solid-Phase Extraction (SPE) methods must use an internal standard with similar chemical properties (i.e. acidic) but this may not be true for solvent extraction unless a back